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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/954,483	09/17/2001	Christian Siebel	RMES-02	6505

7590 05/04/2004

DELTAGEN, INC.
740 Bay Road
Redwood City, CA 94063

EXAMINER

LEFFERS JR, GERALD G

ART UNIT	PAPER NUMBER
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1636

DATE MAILED: 05/04/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary

Application No.

09/954,483

Applicant(s)

SIEBEL ET AL.

Examiner

Gerald G Leffers Jr., PhD

Art Unit

1636

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 17 February 2004.
- 2a) ☐ This action is FINAL. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-10 and 12-31 is/are pending in the application.
- 4a) Of the above claim(s) 27 and 28 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-10, 12-26 and 29-31 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- ☒ Notice of References Cited (PTO-892)
- ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date _____
- 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: _____

DETAILED ACTION

Receipt is acknowledged of an amendment, filed 2/17/2004, in which claims were amended (claims 1, 8, 18, 20-26, 29-31) and in which claim 11 was cancelled. Claims 1-10, 12-31 are pending in the instant application, with claims 27-28 withdrawn from consideration as being directed to a nonelected invention.

Any rejection of record in the office action mailed 8/11/2003 not addressed herein is withdrawn. This action is not final as there are new grounds of rejection presented herein that were not necessitated by applicants' amendment of the claims in the response filed 2/17/2004.

Sequence Compliance

Receipt is acknowledged of applicants' submission on 2/17/2004 of a paper copy of the sequence listing, CRF and corresponding attorney's statements concerning the content of the submitted documents. The application is now in sequence compliance.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-10, 12-26 and 29-31 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the

Art Unit: 1636

claimed invention. **This rejection is maintained for reasons of record in the office action mailed 8/11/2003 and repeated below. The grounds of rejection are extended to amended claim 26 as claim 26 comprises embodiments where the targeting vector of claim 1 has been inserted into the target chromosome via random integration.**

Each of the claims features a targeting construct comprising a positive selection marker, two regions of homology to a target sequence and a “regulator” that controls expression of the positive selection marker. The specification describes the regulator as being “...a sequence or sequences (i.e. polynucleotide sequence or protein sequence) that regulates or controls expression of the selectable marker...” (page 8, lines 15-18). The specification also teaches that the regulator functions to down regulate expression of the selectable marker on the targeting construct when the construct is randomly incorporated into the target genome by illegitimate recombination events (e.g. pages 5-6, bridging paragraphs). This allows the skilled artisan to select for the presence of the positive selection marker and reduce the number of false-positives for proper incorporation of the targeting construct into the target sequence due to the reduced expression of the marker in those cells where the marker is randomly incorporated. Due to this feature, the skilled artisan does not need to utilize negative selection methodologies. The rejected claims encompass an enormous genus of targeting constructs comprising a “regulator” comprising literally any protein or DNA sequence, or combination thereof, arranged in any fashion on the targeting construct. The “regulator” must function, however, to down regulate expression of the positive selection marker if the targeting construct does not insert into the target sequence.

The specification describes a single relevant working example where the two sequences with homology to the target sequence flank a selectable marker cassette and where a gene encoding a transcriptional repressor (*lacI*) is located on the construct on the other side of one of the two targeting sequences from the positive selection marker (e.g. *neo^r*). The gene encoding the selectable marker in this case is under the control of a promoter comprising the cognate operator sequence (*lacO*) for the repressor such that, if random incorporation of the entire targeting construct into the host genome occurs, expression of the positive selection marker is repressed. A double crossover event between the targeting sequences on the construct and the target sequence in the genome, however, results in the release of at least part of the “regulator” and allows more efficient expression of the selectable marker. No other arrangement of the different components of the targeting constructs is described in the instant specification. For example, no description is provided for an alternate arrangement of the two regions of homology to the target sequence and the positive selection marker. The specification asserts that a transcriptional silencer element (e.g. *NRF*, *COL4*, etc.) could also work in *cis* to accomplish the same effect, but no arrangement of such an element has been described in the instant specification. Thus, the instant specification does not provide a basis for one of skill in the art to envision a sufficient number of other arrangements of the recited elements to describe the broadly claimed targeting vectors embraced by the rejected claims.

The prior art does not appear to teach a system of utilizing a “regulator” to down regulate expression of a positive selection marker in targeting constructs when the constructs are randomly inserted into the genome of a host cell. Therefore, the prior art does not offset the deficiencies of the instant specification concerning a basis for one to envision a number of

Art Unit: 1636

alternative arrangements of the recited elements or other types of regulators sufficient to describe the broadly claimed genus.

Given that the term “regulator” apparently encompasses a huge number of possible DNA sequences and proteins sequences, or combinations thereof, and given the functional limitations of what the “regulator” must accomplish, the skilled artisan would not be able to envision a sufficient number of embodiments of the claimed invention to describe the broadly claimed genus of targeting vectors. Therefore, the skilled artisan would reasonably have concluded applicants were not in possession of the claimed invention at the time of filing.

Response to Arguments

Applicant's arguments filed in the response of 2/17/2004 have been fully considered but they are not persuasive. The response essentially argues: 1) the amendment to the claims more clearly defines the organization of the “regulator” in the targeting construct of the claims and makes clear the structural/functional properties of the regulator, and 2) pages 12-14 provide many examples of regulators and selectable markers.

A single embodiment where a selectable marker is put under the control of a bacterial repressor/operator binding site cannot be considered as providing sufficient structural/functional correlation such that the skilled artisan could envision a sufficient number of other embodiments to describe the broadly claimed genus of regulator/selectable marker constructions. As indicated in making the rejection, the recitation of elements that might be usable together provided by the specification does not provide a structural/functional basis for the skilled artisan to envision those embodiments that would actually function in the manner described in the specification for the targeting constructs of the invention. For example, not a single actual embodiment, prophetic

Art Unit: 1636

or otherwise, is described where a transcriptional silencer is the regulator and present in a construct as currently claimed with a particular selectable marker and particular targeting sequences. In addition, the term “regulator” encompasses other embodiments where the “regulator” is not a bacterial repressor or transcriptional silencer (e.g. a transcriptional anti-terminator, a ribozyme specific for the selectable marker, etc.). For these reasons, the skilled artisan would not have been able to envision a sufficient number of specific “regulator”/targeting sequence/selectable marker combinations to describe the broadly claimed genus of constructs bearing these elements.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-25 and 29-31 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Each of the claims recites a limitation of a “regulator”. **This rejection is maintained for reasons of record in the office action mailed 8/11/2003 and repeated here.** The metes and bounds of this term are unclear in the context of the claimed invention. The specification describes the regulator as being “...a sequence or sequences (i.e. polynucleotide sequence or protein sequence) that regulates or controls expression of the selectable marker...” (page 8, lines 15-18). The specification also teaches that the regulator functions to down regulate expression of the selectable marker on the targeting construct when the construct is randomly incorporated into the target genome by illegitimate recombination events (e.g. pages 5-6, bridging

Art Unit: 1636

paragraphs). It is unclear how a regulator can be comprised within a targeting vector and also be a protein. Also, it is unclear whether the term necessarily refers to protein and nucleic acid sequences in certain embodiments. For example, in the embodiment exemplified in the instant specification (e.g. in Figure 5), the targeting vector comprises an operator sequence (lacO) operatively linked to the promoter that drives the selectable marker, as well as a sequence encoding the lac repressor (lacI). In this case, does the term “regulator” refer to the cis-acting lacO sequence, the coding sequence for lacI or the repressor protein; or does it necessarily refer to a combination of all three? It would be remedial to amend the claim language to make clear which elements, protein or DNA sequence or both, must be present in order for a targeting construct to satisfy the limitation of comprising a “regulator”.

Response to Arguments/112 2nd Paragraph

Applicant's arguments filed 2/17/2004 have been fully considered but they are not persuasive. The response essentially argues: the amendment of the claims makes clear what is claimed.

This assertion is not accurate. For example, it remains unclear how a protein can be comprised within the targeting construct as recited. Yet, dependent claims (e.g. claims 12-13) still recite the limitation that the regulator comprises at least one repressor sequence (e.g. a protein?). Therefore, it remains unclear exactly what is being claimed (i.e. a protein coding sequence or a cis-acting structure such as a transcriptional silencer element).

The following are new rejections.

Art Unit: 1636

Claim 1 is vague and indefinite in that there is no clear and positive prior antecedent basis in the claim for the term “the target gene”.

Claims 12 and 13 recite the limitation “wherein the regulator comprises at least one repressor sequence”. It is unclear whether the term “repressor sequence” refers to a coding sequence for a repressor (e.g. the lac repressor), the amino acid sequence of the repressor itself or a repressor binding sequence (e.g. the lac operator). Based upon the positioning of the regulator in the targeting construct it appears the term is intended to specify a repressor coding sequence. It would be remedial to amend the claim to clearly indicate which of the three possibilities is intended.

Claims 20, 22, 29-30 are vague and indefinite in that there is no clear and positive prior antecedent basis for the phrase “the regulator controls expression of a selectable marker” (examiner’s emphasis added). It would be remedial to amend the claims by substituting the word “the” for “a” so that the claims read “the selectable marker”.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claim 26 is rejected under 35 U.S.C. 102(b) as being anticipated by Capecchi et al (AC; U.S. Patent No. 5,627,059; see the entire patent). **This rejection is maintained for reasons of record in the office action mailed 8/11/2003 and repeated below.**

Claim 26 is drawn to an isolated host cell comprising a modification or disruption of a target gene, wherein the target gene is modified or disrupted by insertion of a targeting vector into the host cell.

Capecchi et al teach the use of positive-negative targeting vectors that comprise targeting sequences flanking a positive selection marker and which further comprise a negative selection marker outside of the targeting cassette that allows for selection against random insertion events (e.g. Abstract; Figure 1). The '059 patent teaches examples where particular genes in a target cell have been inactivated by insertion of a targeting construct (e.g. Example 4-Disruption of the *hox1.4* locus in mouse ES cells).

Response to Arguments/35 U.S.C. 102(b)-Capecchi et al

Applicant's arguments filed 2/17/2004 have been fully considered but they are not persuasive. The response essentially argues: the amendment of the claim to depend on claim 1 obviates the rejection as Capecchi et al have not taught the vector of claim 1. This argument is not persuasive due to the fact that if applicants' vector works as it is supposed to function, and the "regulator" does not function by binding to some element of the selectable marker (e.g. it is a "cis-acting" element such as a transcriptional silencer), then the resulting disrupted gene would be essentially indistinguishable from that taught by Capecchi et al due to the absence of any regulator element in the disrupted gene.

Claims 1, 18, 20, 29-30 are rejected under 35 U.S.C. 102(b) as being anticipated by Kuebler et al (J. Mol. Biology, Vol. 281, pages 803-814; see the entire reference). **This is a new rejection.**

Art Unit: 1636

It is noted that the term "selectable marker cassette" is not explicitly defined in the instant specification and can be interpreted broadly to encompass any selectable trait that is, as recited by the claims, flanked by targeting sequences.

Kuebler et al teach the construction of a vector, pDK H436am, which comprises a gene encoding a mutant form of the T4 terminase (i.e. gp17) under control of the T7 RNA polymerase promoter (i.e. a "regulator" of expression of the selectable marker) of pET 9D (e.g. column 2, page 807). In this case, the "selectable marker cassette" is the H436am mutation, which comprises sequences on either side of it homologous to gene 17 of the wildtype phage. Kuebler et al cross the H436am mutation into a 17amK166 phage to generate recombinant phage comprising the selectable H436am mutation and lacking the 17amK166 mutation (i.e. 17amK166 mutants are unable to replicate in the genetic background used for selection). Recombinant phage were then selected based upon their ability to grow on the His suppressor background but not on the equivalent suppressor minus strain of *E. coli* (e.g. page 807, second full paragraph of column 2).

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Gerald G Leffers Jr., PhD whose telephone number is (571) 272-0772. The examiner can normally be reached on 9:30am-6:00pm.

Art Unit: 1636

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Remy Yucel can be reached on (571) 272-0781. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Gerald G Leffers Jr., PhD
Primary Examiner
Art Unit 1636

ggl


GERRY LEFFERS
PRIMARY EXAMINER

Notice of References Cited

Application/Control No.

09/954,483

Applicant(s)/Patent Under
Reexamination
SIEBEL ET AL.

Examiner

Gerald G Leffers Jr., PhD

Art Unit

1636

Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kuebler, et al. "Functional Analysis of the DNA-Packaging/Terminase Protein gp17 from Bacteriophage T4", Journal of Molecular Biology, Vol. 281, pages 803-814, 1998.
	V	
	W	
	X	

A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

JMB

Functional Analysis of the DNA-Packaging/Terminase Protein gp17 from Bacteriophage T4

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In bacteriophage T4, the terminase complex constituted by the large subunit gp17 (69 kDa) and the small subunit gp16 (18 kDa) is a critical component of the ATP-driven DNA-packaging pump that translocates DNA into an empty capsid shell. Evidence suggests that the large subunit gp17 is the critical component and consists of a number of the functional sites required for DNA-packaging. It exhibits a terminase activity that introduces non-specific cuts into DNA, a portal vertex binding site that allows linkage of cleaved DNA to an empty prohead, an *in vitro* DNA-packaging activity, and an ATPase activity. In addition, a consensus metal-binding motif and two consensus ATP-binding sites have been identified by sequence analysis. In order to understand the mechanism of action of the multifunctional gp17, we developed an expression-based selection strategy to select for mutants that are defective in terminase function. Characterization of one of the mutants revealed a unique phenotype in which a single H436R mutation resulted in a dramatic loss of both the terminase and the DNA-packaging functions. Indeed, *in vivo* substitution of H436 with any of the 12 amino acids for which a suppressor is available was lethal to T4 development. According to one hypothesis, H436 is part of a metal-binding motif that is essential for gp17 function. This hypothesis was tested by introducing mutations at each of the three histidine pairs, the H382-X₂-H385 pair, the H411-X₂-H414 pair and the H430-X₅-H436 pair, which constitute the histidine-rich region near the C terminus of gp17. A mutation at either the H411 pair or the H430 pair resulted in a loss of gp17 function, whereas a mutation at the H382 pair had no effect. In addition to the putative metal-binding motif, substitutions at residue K166 within the putative N terminus-proximal ATP-binding site also resulted in a loss of gp17 function. We propose that a metal-binding motif involving the histidine residues within the sequence H411-X₂-H414-X₁₅-H430-X₅-H436 is essential for gp17 function. Metal-terminase interactions may be required for structural alignment and stabilization of functional sites in phage T4 terminase and other double-stranded DNA phage terminases.

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Keywords: bacteriophage T4; DNA-packaging; terminase; metal-binding site; ATP-binding site

*Corresponding author

Introduction

Double-stranded (ds) DNA-packaging in icosahedral bacteriophages is a complex biological process

Abbreviations used: am, amber, ds, double-stranded; g, gene; gp, gene product; m.o.i., multiplicity of infection; PCR, polymerase chain reaction; p.f.u., plaque-forming units; SOE, splicing by overlap extension; IPTG, isopropyl- β -D-thiogalactopyranoside.

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involving (i) recognition of the complex concatemeric DNA substrate, (ii) cutting of DNA and initiation of packaging, (iii) translocation of one unit length genome into the capsid shell, (iv) organization of DNA inside the capsid and (v) cutting of DNA and termination of packaging (see Earnshaw & Casjens, 1980; Casjens, 1985; Feiss, 1986; Black, 1988). While it is clear that ATP hydrolysis drives DNA-packaging, the basic molecular mechanisms of DNA translocation and chromosome packing inside the capsid shell are still a mystery. Genetic and biochemical studies

suggest that a DNA-packaging pump, constituted by the capsid shell protein, the portal vertex protein, and two non-structural terminase proteins, drives DNA-packaging (Black, 1995). While there is no apparent homology at the amino acid sequence level, remarkably, the basic structural and functional features of the packaging components are conserved among all the dsDNA bacteriophages, suggesting a common mechanism (Casjens, 1985; Black, 1995).

The bacteriophage T4 terminase, analogous to the other phage terminases, is constituted by one large subunit, the 69 kDa gp17, and one small subunit, the 18 kDa gp16 (Rao & Black, 1988; Powell *et al.*, 1990). However, the stoichiometry of the subunits in the holoterminase complex is unknown. Recently, we discovered that the large subunit gp17 alone can catalyze a number of functions involved in packaging DNA (Rao & Black, 1988; Bhattacharyya & Rao, 1993, 1994; Rao & Leffers, 1997). When expressed in *Escherichia coli*, gp17 cleaved both the plasmid DNA and the *E. coli* genomic DNA in a non-specific manner† (Bhattacharyya & Rao, 1993, 1994). The purified gp17 is also highly active for packaging DNA *in vitro* (Rao & Black, 1988; Leffers & Rao, 1996). It also exhibited an ATPase activity, hydrolyzing ATP to ADP and P_i (Rao *et al.*, 1996; and unpublished results). The small subunit gp16, on the other hand, did not exhibit any of these activities, but it did enhance the *in vitro* DNA-packaging and the ATPase activities associated with gp17 (Rao & Leffers, 1997; and unpublished results). Recent data suggest that gp16 binds to DNA and, analogous to the small terminase subunits from the other dsDNA phages, it may be required for recognition of DNA followed by initiation of packaging (Lin *et al.*, 1997; L. Black, personal communication).

Clearly, the large terminase subunit gp17 is a critical component of the DNA-packaging pump and, quite possibly, the one that couples ATP hydrolysis to DNA translocation. Structural and functional characterization of gp17 may shed light on the role of this protein in the ATP-coupling process and the mechanistics of DNA translocation. In this study, we have discovered that a histidine-rich region, the putative metal-binding motif near the C terminus, and a consensus ATP-binding motif near the N terminus (Guo *et al.*, 1987), are essential for gp17 function. Missense mutations at certain histidine residues within the putative metal-binding motif resulted in a dramatic loss of both the terminase and the DNA-packaging functions. The data suggest that metal-terminase interactions are critical to the structure and function of terminases

from phage T4 and other dsDNA bacteriophages (Guo *et al.*, 1987; Morita *et al.*, 1994).

Results

A selection system for phage T4 terminase mutants

As indicated in Introduction, we have previously shown that a nuclease activity is associated with the large terminase subunit gp17 (Bhattacharyya & Rao, 1993). Expression of gp17 in *E. coli* from the phage T7 promoter resulted in extensive cleavage of both the plasmid DNA and the *E. coli* genomic DNA, generating a characteristic smear that extended throughout the lane upon agarose gel electrophoresis (compare the uninduced lane 1 with the induced lanes 2 to 4 in Figure 1A). We referred to this activity as the terminase activity because, consistent with the apparent circularly permuted nature of the phage T4 mature DNA ends, the enzyme cleaved DNA in a non-specific manner (Bhattacharyya & Rao, 1993); this is consistent with the fact that, in all the dsDNA phage terminases examined, the terminase activity is associated with the large terminase subunit (Black, 1988).

An important observation, which is consistent with the gp17-associated terminase activity, was that a high basal level expression of gp17 was lethal to *E. coli*. This was evident from a number of experiments. First, it was not possible to transform and maintain the T7 promoter-controlled gp17 constructs in the expression strain *E. coli* BL21(DE3). This strain is known to produce significant amounts of T7 RNA polymerase from the leaky *lacUV5* promoter, producing high basal levels of foreign gene products (Studier *et al.*, 1990). Indeed, the transformation efficiency of pR17-8 (a wild-type gp17 cloned under the T7 promoter control; Bhattacharyya & Rao, 1993) into *E. coli* BL21(DE3) was ~10⁵-fold less than that into the isogenic non-expression strain *E. coli* BL21 (Table 1). On the other hand, a mutant plasmid (pR17Δ5) that expressed a defective gp17 with a 77 amino acid residue truncation at the C terminus could be transformed as efficiently into BL21 (DE3) as into BL21 (Bhattacharyya & Rao, 1993; data not shown). The pR17-8 can, however, be successfully transformed into the expression strains BL21 (DE3) pLys-S or BL21 (DE3) pLys-E, which are isogenic to BL21 (DE3) except that these strains produce reduced basal levels of functional T7 RNA polymerase (Studier *et al.*, 1990). Also, consistent with the fact that pLys-S generates higher basal levels of T7 RNA polymerase than pLys-E, the pR17-8 transformant colonies with pLys-S were much smaller than those with pLys-E. Finally, we observed an abundant presence of filamentous *E. coli* in the uninduced BL21 (DE3) pLys-S/pR17-8 cultures, which expressed low but significant levels of gp17, but not in the control BL21/pR17-8 cultures (isogenic non-expression strain), or in the BL21 (DE3)

† The DNA cleavage activity is referred to as the terminase function, whereas the head-filling activity is referred to as the DNA-packaging function. However, in some contexts, terminase and DNA-packaging protein are also used as general terms in reference to gp16 and gp17 subunits.

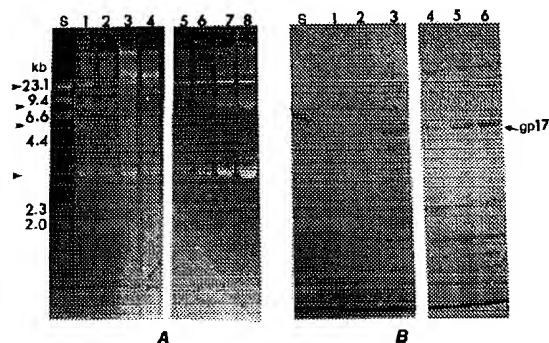


Figure 1. The mutant pDK 2.1 exhibits no terminase activity. *E. coli* cells carrying the wild-type pR 17-8 plasmid or the mutant pDK 2.1 plasmid were grown at 37°C in LB medium containing the appropriate antibiotic(s) to about 4×10^8 cells/ml. An aliquot of this culture (uninduced culture) was withdrawn and was divided into halves. IPTG was then added to the remaining culture to a final concentration of 0.4 mM. Aliquots were withdrawn at the indicated time-points after IPTG addition (induced cultures), and were divided into halves. One half was analyzed for DNA cleavage (Figures 1A and 2A; Table 2) and the other half was analyzed for gp17 expression (Figures 1B and 2B). A, EtBr-stained agarose gel showing the terminase activity. Miniprep DNAs were prepared by the alkaline lysis procedure of Birnboim & Doly (1979) from *E. coli* BL21 (DE3) pLys-S carrying either pR17-8 (lanes 1 to 4) or the mutant pDK 2.1 (lanes 5 to 8). The cells were either from uninduced cultures (lanes 1 and 5) or from cultures induced with IPTG for 30 minutes (lanes 2 and 6), 1.5 hours (lanes 3 and 7), or 2.5 hours (lanes 4 and 8). The samples corresponding to an equivalent culture volume (about 200 μ l) were loaded into each well of an 0.8% agarose gel containing 1 μ g/ml EtBr and were electrophoresed in TBE buffer (Maniatis *et al.*, 1982). Lane S shows HindIII-digested λ DNA as size markers. The arrows on the left indicate the positions of the plasmid DNA bands (the same is applicable to Figure 2). B, Coomassie blue-stained SDS-10% PAGE showing the expression of gp17. The samples were prepared for SDS-PAGE from *E. coli* BL21 (DE3) pLys-S carrying either the pR 17-8 (lanes 1 to 3) or the mutant pDK 2.1 (lanes 4 to 6) by the procedure described by Laemmli (1970). Lanes 1 and 4, uninduced cultures; lanes 2 and 5, cultures after 30 minutes induction with IPTG; lanes 3 and 6, cultures after 2.5 hours induction with IPTG. Samples in each lane correspond to an equivalent culture volume (about 100 μ l). Lane S shows purified gp17 used as the molecular size standard. The position of gp17 band is indicated on the right with an arrow.

pLys-s/pR17 Δ 5 cultures, which express the truncated gp17. The filaments presumably arose as a result of DNA damage caused by gp17 cleavage of *E. coli* genomic DNA; this may have triggered SOS responses, thereby inducing *SulA* expression and inhibition of septation (Gottesman *et al.*, 1981).

Based on these observations, we argued that, if the terminase-dependent DNA cleavage was lethal to *E. coli*, transformability into BL21 (DE3) can be used as a selection pressure for selecting mutants

Table 1. Efficiency of transformation of g17 constructs into *E. coli* BL21(DE3)

Plasmid	Transformants per μ g DNA
pET 9D (vector alone)	2.8×10^6
pR 17-8	<20
pDK 2.1	1.8×10^6
pDK H436R	1.1×10^6
pDK E536V	<40

The plasmid DNAs were prepared from the non-expression strain *E. coli* BL21, which maintained each of the plasmids in a stable state. Equivalent amounts of each DNA were used for transformation by electroporation. In the control transformations, i.e. transformation of each of the plasmids back into BL21, the efficiencies of transformation were approximately the same (data not shown).

that are defective in terminase function. As the evidence would show below, this was indeed the case, since virtually every transformant that originated upon transformation of mutated pR17-8 into BL21(DE3) was terminase-defective (A. Robichaux & V. B. R., unpublished data). An analogous system was used for the isolation of phage λ terminase-defective mutants (Murialdo, 1988; Davidson & Gold, 1992).

Isolation of terminase-defective mutants

The basic strategy for terminase mutant selection involved (i) random mutagenesis of pR17-8 plasmid with nitrous acid, which is known to predominantly introduce transitions (Schuster, 1960), (ii) amplification by PCR of the 942 bp 3'-portion of the mutated g17 (the complete g17 sequence is 1830 bp long), (iii) replacement of the 3'-portion of the wild-type g17 with the mutated DNA fragment, and (iv) selection of terminase-defective mutants by transformation into *E. coli* BL21 (DE3); see Materials and Methods. The 3'-portion of g17 was chosen because there was prior evidence to suggest that the C terminus of gp17 is essential for terminase function (Bhattacharyya & Rao, 1993). The mutagenesis conditions were optimized such that, on average, there would be one mutagenesis event per 800 bp of random DNA. About 60 colonies, which, by their large size, appear to be terminase-defective mutants, were obtained. Miniprep plasmid DNAs were prepared from a number of colonies selected at random, and the DNAs were re-transformed into BL21 (DE3) pLys-S. This was important because, for analysis of the putative terminase mutants, the genetic background should be the same as the wild-type pR17-8 control (as discussed above, pR17-8 cannot be maintained in the BL21 (DE3) background).

We anticipated the recovery of three types of mutants: (i) missense mutants that would have either lost the terminase activity, or expressed a defective, though non-lethal, terminase activity; (ii) expression-defective mutants that would express very low basal levels of gp17; and (iii) nonsense, frameshift or deletion mutants that would have lost the terminase activity. As the class (i) mutants

are the most interesting for functional analysis, the mutants were screened by multiple approaches to eliminate those belonging to classes (ii) and (iii). These approaches included: (i) *in vivo* terminase assay to assess an alteration or a loss of the DNA cleavage activity; (ii) SDS-PAGE of total cell extracts before and after IPTG induction to assess the relative efficiency of gp17 expression and to detect truncations and deletions; and (iii) DNA sequencing to precisely identify the mutated site(s). Roughly half of the mutants analyzed turned out to have large deletions in g17. One of the mutants (pDK 2.1) that showed no apparent deletion, and overexpressed a full-length gp17 at levels comparable with the wild-type gp17 (Figure 1B), was selected for detailed analyses.

The mutant pDK 2.1, unlike the wild-type pR17-8, exhibited no terminase activity. The appearance of a DNA smear throughout the lane, a hallmark of T4 terminase activity, was not observed with this mutant (lanes 5 to 8, Figure 1A). On the other hand, the intensity of the plasmid bands increased during induction, presumably due to an increase in cell number; this was observed previously with the negative controls and with mutant clones that do not express a functional terminase (Bhattacharyya & Rao, 1993). Consistent with the lack of terminase activity, the mutant pDK 2.1 DNA, unlike the wild-type pR17-8 DNA, can be transformed into *E. coli* BL21 (DE3) as efficiently as into BL21 (Table 1). DNA sequencing of the entire 770 bp mutated region of pDK 2.1 showed two single point mutations, an A → G transition at position 1307 that resulted in an H → R conversion at residue 436, and an A → T transversion at position 1607 that resulted in a E → V conversion at residue 536.

A single H436R mutation is responsible for the loss of terminase activity

The presence of two mutations in the terminase-defective mutant pDK 2.1 would be consistent with three hypotheses: (i) both the mutations are needed to confer the terminase defect; (ii) only one of the mutations is needed to confer the defect, while the second mutation is a silent mutation; or (iii) each mutant by itself can confer only a partial defect but both together will have an additive (or synergistic) effect. Discrimination between these hypotheses is necessary to identify the site(s) essential for terminase function.

Two independent mutants, one with the H436R mutation and the other with the E536V mutation, were constructed by site-directed mutagenesis using a PCR-based "splicing by overlap extension" (SOE) strategy. Unlike the conventional mutagenesis strategies, SOE allowed direct incorporation of the desired mutation(s) into g17 DNA by using synthetic mutant oligonucleotides (see Materials and Methods). The mutagenesis strategy was designed in such a way that it would incorporate the desired mutation as well as a silent mutation(s)

at a nearby sequence, which would either create a new restriction site, or eliminate an existing restriction site. The mutants were first selected based on a change in this restriction site, and were then sequenced to confirm the presence of the appropriate missense mutation and absence of any non-specific mutations. The mutant constructs were then transformed into the permissive strain BL21 (DE3) pLys-S.

Transformants for each mutant were randomly selected and tested for (i) expression of gp17, and (ii) terminase activity. As shown in Figure 2B, both the mutants (pDK H436R and pDK E536V) expressed gp17 to approximately the same level as pR17-8. But, the H436R mutant exhibited no terminase activity (compare H436R lanes 4 to 6 with the wild-type lanes 1 to 3, Figure 2A), whereas the E536V mutant exhibited the same level of terminase activity as the wild-type (compare E536V lanes 7 to 9 with the wild-type lanes 1 to 3, Figure 2A). Consistent with these results, H436R mutant DNA could be efficiently transformed into BL21(DE3), whereas the E536V mutant DNA could not be transformed into BL21(DE3) (Table 1). These data clearly demonstrated that the loss of terminase function in pDK 2.1 was due to a single H436R mutation, whereas the E536V mutation was a silent mutation.

Does the H436R mutation also result in a DNA-packaging defect?

To test whether the terminase-defective mutation has any effect on the DNA-packaging function, *in vitro* DNA-packaging assays were performed.

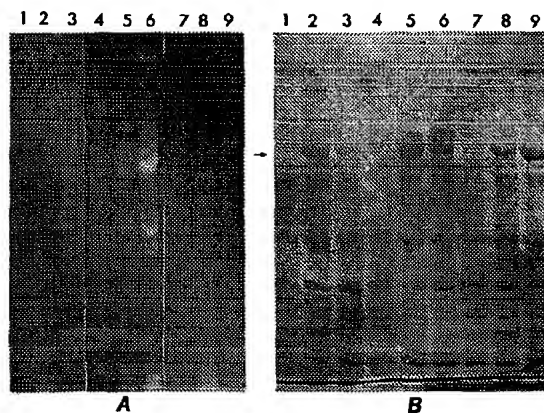


Figure 2. A single H436R mutation is responsible for the loss of terminase activity. The basic experimental design is the same as that in Figure 1. See the legend to Figure 1 for details. A, EtBr-stained agarose gel. B, Coomassie blue-stained SDS-10% PAGE. Lanes 1 to 3, pR17-8; lanes 4 to 6, pDK H436R mutant; lanes 7 to 9, pDK E536V mutant. Lanes 1, 4 and 7, uninduced cultures; lanes 2, 5 and 8, cultures after 30 minutes induction with IPTG; lanes 3, 6 and 9, cultures after two hours induction with IPTG. The arrow indicates the position of the gp17 band.

The *in vitro* system is particularly suited to address this question, because the packaging substrate used was an already cut mature T4 DNA. This means that the DNA-packaging activity of the mutant protein was determined independent of the cleavage activity, since the terminase function is not a necessary requirement for packaging mature DNA. Indeed, mutants of this sort that lost terminase activity but retained the mature DNA-packaging activity have been reported for phage λ and phage T3 terminases (Hwang & Feiss, 1996; Morita *et al.*, 1994).

All the three gp17 mutant proteins, the original double mutant pDK 2.1, the H436R mutant, and the E536V mutant, were tested for *in vitro* packaging of phage T4 DNA. Equivalent amounts of lysates from IPTG-induced cultures were added to the *in vitro* packaging reaction mixture and the p.f.u. formed were determined. The data showed that the mutants pDK 2.1 and pDK H436R both of which have the H436R mutation in common exhibited no *in vitro* DNA-packaging activity (Table 2); even the addition of excess lysate did not show any detectable packaging activity (data not shown). On the other hand, the mutant pDK E536V exhibited as high a DNA-packaging activity as the wild-type pR 17-8 control (Table 2). These data clearly showed that the H436 residue is also critical for the DNA-packaging function, whereas the E536V mutation is a silent mutation.

Table 2. Terminase and *in vitro* DNA-packaging activities of gp17 mutants

Construct	Terminase activity ^a	Phage yield (p.f.u./reaction mix) ^b
pET 9D (vector alone)	—	$<2 \times 10^2$
Partially purified gp17 (from pR 17-8)	—	2.3×10^6
pR 17.8	+++	1.5×10^6
pDK 2.1	—	$<2 \times 10^2$
pDK H436R	—	$<2 \times 10^2$
pDK E536V	+++	1.4×10^6
pDK H382R	+++	4.7×10^6
pDK H414R	—	$<2 \times 10^2$
pDK H430R	+	3.3×10^5
pDK K166L	—	$<2 \times 10^2$

^a Terminase activity was determined according to the procedure described in the legend to Figure 1.

^b The *E. coli* BL21 (DE3) pLys-S containing the cloned 17 construct were grown in LB medium (400 ml) at 30°C to $\sim 4 \times 10^8$ cells/ml. The cells were induced with 0.4 mM IPTG for one hour. The cells were harvested by centrifugation at 6000 rpm for ten minutes. The pellet was resuspended in 10 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM ATP, 5 mM Mg²⁺, 3 mM β -mercaptoethanol and 0.5 mM EDTA. The cells were lysed by two cycles of French-pressing and the cell debris was removed by centrifugation at 15,000 rpm for ten minutes. A 2 to 10 μ l aliquot of the supernatant was then tested for the *in vitro* DNA-packaging activity according to the procedure described by Rao & Black (1988). A freshly prepared 17amrII-infected extract was used as the source of empty capsids, tails and other phage assembly factors.

The H436R mutation also exhibited a DNA-packaging defect *in vivo*

Does the H436R mutation also exhibit a DNA-packaging defect if expressed during a T4 infection, or is it somehow related to overexpression of the mutant protein in *E. coli*? This is an important point to clarify, since the behavior of a protein in an artificial environment, such as the overexpression in *E. coli*, may not be the same as that during the normal course of T4 development (Mitraki & King, 1989). Even if H436R gp17 does indeed exhibit a DNA-packaging defect *in vivo*, is it a lethal defect or a partial defect? A partial defect would imply that the mutant phage would retain the ability to form plaques.

These questions can be addressed only by transferring the H436R mutation into the phage T4 genome. Since the H436R mutation has a high probability of being lethal, it would be unlikely that a simple marker rescue strategy would successfully recover the mutation into the phage T4 genome (Mattson *et al.*, 1977). We therefore took a suppressor-based genetic approach (Kleina *et al.*, 1990). A mutant g17 clone, pDK H436am, was first constructed by introducing (i) an amber termination codon at the H436 position, and (ii) a new restriction site (*EcoRV*) at a nearby site, using the SOE mutagenesis strategy. The amber mutation was then transferred into the phage T4 genome by infecting the BL21/pDK H436am cells with 17amK166. The progeny phage, some of which will have the H436am mutation as a consequence of recombination between the T4 genome and the mutant plasmid, were plated on an *E. coli* strain expressing the histidine suppressor (Kleina *et al.*, 1990). The 17amK166 mutant was chosen for infection because we found that the K166 mutation could not be suppressed by the histidine suppressor, and hence there would be no background p.f.u. from the parental phage (K166 corresponds to lysine 166 within the consensus ATP-binding site, see below). The plaques generated should be either the wild-type phage or the H436am mutant phage. Individual plaques were screened and the H436am plaques were selected based on their phenotypic characteristics, i.e. the H436am phage, unlike the wild-type phage, should form plaques on the *E. coli*-His suppressor strain, but not on the suppressor-minus *E. coli* P301. Several plaques with such a phenotype were selected and purified. The presence of the amber mutation at the H436 position and the *EcoRV* restriction site in the adjacent region were confirmed by PCR amplification and DNA sequencing; no additional secondary mutation was detected in the neighboring region.

The suppressor strategy now allows testing the effect of substituting H436 with any of the 12 amino acids using an appropriate *E. coli* suppressor strain. This was done (i) qualitatively by spot-testing a number of randomly selected H436am plaques on each of the suppressor strains, and (ii)

quantitatively by titration of phage stocks on each of the suppressor strains. None of the T4.H436am isolates generated plaques on any of the 12 suppressor strains tested (Gly, Ala, Leu, Ser, Cys, Glu, Gln, Arg, Pro, Lys, Phe and Tyr) other than the homologous *E. coli*-His suppressor strain. The quantitative data further showed that the plating efficiency of the T4.H436am phage (p.f.u. on a given suppressor strain/p.f.u. on *E. coli*-His suppressor strain) was $<10^{-8}$ for any of the 12 suppressor strains tested. Electron microscopy of the lysates from the H436am-infected *E. coli*-Arg suppressor strain showed that $>95\%$ of the proheads in these extracts are empty proheads, the classical packaging-defective phenotype observed in 17am mutants (Figure 3A). On the other hand, lysates from the T4.H436am-infected *E. coli*-His suppressor strain showed that $>50\%$ of the heads are DNA-full heads (Figure 3B).

The above data suggest that any of the 12 amino acid substitutions at H436, even when expressed during phage T4 infection, resulted in a DNA-packaging defect and were lethal to T4 development. These data would strongly support the hypothesis that the H436 residue is critically required for gp17 function. In conclusion, H436 is a critical residue and substitutions at this residue resulted in a severe loss of both the terminase and the packaging functions, a unique phenotype that has not yet been reported with the other phage terminases.

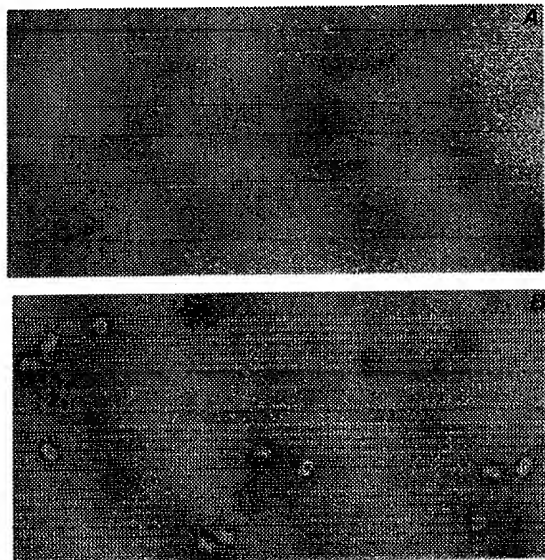


Figure 3. The mutant H436R is defective for DNA-packaging *in vivo*. *E. coli* cells expressing either the arginine suppressor (A) or the histidine suppressor (B) were infected with the mutant T4.H436am phage at an m.o.i. of 5. Thirty minutes after infection at 37°C, cell lysates were prepared and were negatively stained with uranyl acetate. The samples were observed under a Zeiss electron microscope at a magnification of 30,000x.

Amino acid substitutions at additional histidine residues within the histidine-rich region resulted in a loss of gp17 function

We proposed three hypotheses that could explain the critical nature of the H436 residue. First, the H436 residue is part of an essential metal-binding motif and substitutions at this residue resulted in the disruption of metal-gp17 interactions. Second, the H436 residue is part of the gp17 active site and is directly involved in the catalysis of DNA cleavage and translocation. Third, the H436R mutation resulted in a major structural defect in gp17 resulting in abortive folding and loss of function.

Of these hypotheses, the third hypothesis appears to be unlikely, since an H \rightarrow R substitution is predicted to be a conservative one from a structural standpoint and, moreover, it is unlikely that all the 12 amino acid substitutions would have resulted in a major structural/folding defect. With regard to the first hypothesis, if the H436 is part of a metal-binding motif, such a motif would be constituted by one or more additional histidine residues (Berg, 1986; Regan, 1995). A putative metal-binding motif has been identified in gp17 and other phage terminases (Guo *et al.*, 1987; Figure 4). The prediction therefore would be that mutations at additional histidine residues within this motif should also result in phenotypes similar to that at H436. If, on the other hand, the second hypothesis were correct, i.e. if the H436 residue has a catalytic role, it is expected to be a critical residue and it is unlikely that mutations at the additional histidine residues would result in a loss of function.

In order to discriminate among the above hypotheses, we introduced an H \rightarrow R mutation at each of the three nearby histidine pairs (the H382-X₂-H385 pair, the H411-X₂-H414 pair, and the H430-X₅-H436 pair) and asked how the mutation affected the terminase and the packaging functions. An H \rightarrow R mutation was introduced at residue H382 of the first histidine pair, at H414 of the

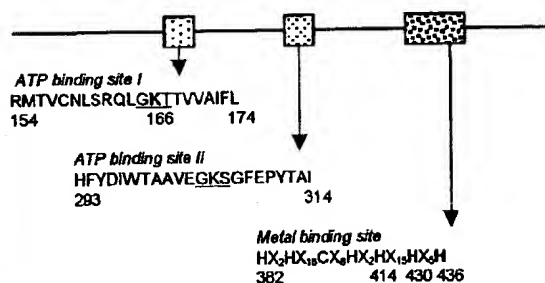


Figure 4. A representation of gp17 with the proposed motifs. The sequences corresponding to the ATP-binding sites are as proposed by Guo *et al.* (1987). The metal-binding motif is as proposed by Guo *et al.* (1987), except that it included an additional histidine pair (H430-X₅-H436). The residues shown in bold are those found to be critical for gp17 function in this study.

second histidine pair, and at H430 of the third histidine pair. All the mutants were constructed by the SOE strategy, and the presence of the newly created mutation and a change in the restriction site in the nearby sequence were verified by DNA sequencing.

The three mutant constructs (pDK H382R, pDK H414R and pDK H430R) were transformed into BL21(DE3) pLys-S, and the level of gp17 expression, terminase activity and *in vitro* DNA-packaging activity were determined using pR 17-8 as the positive control and pDK 2.1 as the negative control. SDS-PAGE showed that all the mutants overexpressed gp17 to roughly the same levels (data not shown). But, with respect to the terminase activity, the mutant pDK H382R showed no loss of terminase activity, the mutant pDK H414R showed no significant terminase activity, and the mutant pDK H430R showed a reduced terminase activity (Table 2). The *in vitro* DNA-packaging activities correlated with the terminase activities; the pDK H382R mutant packaged the mature DNA with about the same efficiency as the wild-type pR17-8, the mutant pDK H414R showed no detectable *in vitro* DNA-packaging activity, and the pDK H430R mutant showed about fivefold lower packaging activity than that of the wild-type pR17-8 (Table 2).

These data show that more than one histidine residue within the histidine-rich region is essential for gp17 function. This is consistent with the metal-binding site hypothesis, although the catalytic hypothesis cannot be ruled out.

Mutations in the consensus ATP-binding site also resulted in a loss of gp17 function

An interesting observation from the above analyses is that the mutants showed correlated effects on both the terminase and the DNA-packaging functions, i.e. mutants that showed a loss of terminase function also showed a corresponding loss of DNA-packaging function (H414R, H430R and H436R), and the mutants that showed no loss of terminase function also retained the full complement of the DNA-packaging function (H382R and E536V). Does this suggest a communication among the functional sites in gp17 terminase? Would mutants in DNA-packaging (which may also disrupt such communications) also show defects in terminase function? While it is an undoubtedly complex issue, the data might support a functional linkage between the functional sites in terminases. Mutants in the consensus ATP-binding site, which is presumably involved in DNA translocation (Morita *et al.*, 1994), are good candidates to address this question. These experiments should also clarify the functional significance of the proposed consensus ATP-binding motifs in gp17 (Guo *et al.*, 1987; see Figure 4). We chose the putative N-terminal ATP-binding site and introduced an amber mutation at the critical K166 position by the same strategies described

above for the H436am mutant. For testing the effect of amino acid substitutions at the K166 residue, we converted a number of *E. coli* suppressor strains into (DE3) pLys-S strains by lysogenization with λ (DE3) followed by transformation with the pLys-S plasmid (Studier *et al.*, 1990). Of these, only the leucine suppressor strain overexpressed the K166L mutant to levels comparable to that of the wild-type pR 17-8 control (data not shown). Analysis of the K166L mutant showed that it exhibited no significant terminase activity and no *in vitro* DNA-packaging activity (Table 2). We have also transferred the K166am mutation into the T4 genome using the strategy described above for the H436am mutation (17amNG178 mutant, which did not plate on *E. coli*-Lys was used for infection). Tests on a number of randomly selected T4.K166am plaques revealed that they do not form plaques on any of the suppressor strains. The plating efficiency (p.f.u. on a given suppressor strain/p.f.u. on *E. coli*-Lys suppressor strain) of the T4.K166am phage was $<10^{-7}$ for any of the 12 suppressor strains tested. Electron microscopy of the lysates from the T4.K166am-infected *E. coli*-Arg suppressor strain showed the classical DNA-packaging defect. Greater than 90% of the proheads were empty proheads; on the other hand, in the control *E. coli*-Lys infections, >40% proheads were DNA-full heads (data not shown).

Discussion

The selection system

A number of observations correlated with the hypothesis that a high basal level expression of gp17 terminase is lethal to *E. coli*. In fact, we found that the gp17 expression was lethal even to the host mutant C41(DE3), which was selected by Miroux & Walker (1996) as a survivor under IPTG-induced, high-level expression of toxic proteins. These observations were relevant to the functionality of gp17 and allowed the development of a novel strategy to select for terminase-defective mutants (Davidson & Gold, 1992). As the phage T7 system is a widely used system for expression of foreign gene products, this approach may be useful as a general strategy for selection of non-toxic (or less toxic) variants of the expressed gene product.

Functional analysis of mutants

We used a combination of experimental approaches to analyze the mutants. These include: (i) transformation into BL21 (DE3), (ii) *in vivo* terminase activity, (iii) *in vitro* DNA-packaging activity, and (iv) suppressor-based *in vivo* DNA-packaging. We believe that such a multi-faceted approach is essential for functional analysis, since each approach has its own limitations. The *in vivo* suppression-based approach deserves comment. It is undoubtedly a powerful approach, since it allowed the substitution of either the H436 or the

K166 residue with any of the 12 amino acids for which a suppressor is available. In addition, since the effect(s) of substitution are evaluated during T4 infection, it has eliminated potential artifacts associated with the *E. coli*-overexpressed gp17. However, the efficiency of suppression is known to vary among different suppressor strains, and in addition, there would be unknown context effects (Kleina *et al.*, 1990). But, unlike the structural proteins, which are required in stoichiometric quantities, the above limitations, if they exist, are somewhat minimized in the case of gp17, which, analogous to the other terminases, is expected to be a processive enzyme acting in a catalytic fashion. Furthermore, the plating efficiencies of the mutants on various suppressors are extremely low (10^{-7} to 10^{-8}) and virtually every rare plaque that appeared on these suppressors turned out to be an amber revertant. Equally important to note, the results from different approaches were in good correlation.

The consensus ATP-binding site

All the current models for DNA-packaging, and the universal requirement for ATP in all the *in vitro* DNA-packaging systems, implicate the coupling of ATP hydrolysis to DNA translocation (Earnshaw & Casjens, 1980; Black, 1988). Consistent with this, the terminase subunits, the large subunit in particular, possess one or more ATP-binding consensus sequences, and an *in vitro* ATPase activity (Walker *et al.*, 1982; Guo *et al.*, 1987; Franklin & Mosig, 1996). gp17 was proposed to possess two A-type ATP-binding consensus sequences (Figure 4). Recent results in our laboratory demonstrated an ATPase activity associated with the highly purified gp17 (Rao & Leffers, 1997; G. Leffers & V.B.R., unpublished results). In this study, substitution of the critical K166 residue within the N-terminal ATP-binding consensus sequence with leucine resulted in a loss of terminase activity and the *in vitro* DNA-packaging activity. Furthermore, *in vivo* substitution with any of the 12 amino acids was lethal to T4 development. Electronmicroscopy of phage lysates in which K166 residue was substituted with the conservative arginine residue showed empty proheads, suggesting a DNA-packaging defect. All these observations are consistent with the hypothesis that the N-terminal consensus ATP-binding sequence is a legitimate ATP-binding motif and is critical to gp17 function. Whether this is the site responsible for the gp17-associated ATPase activity is being investigated.

The proposed metal-binding motif

Since the report by Miller *et al.* (1985), consensus metal-binding motifs have been found in many nucleic acid-binding proteins. In the case of the phage terminases, first Berg (1986), and then Guo *et al.* (1987) identified potential histidine-rich

metal-binding motifs in phages λ , T3, T7, T4 and ϕ 29. The evidence presented here and by Morita *et al.* (1994) argue that these putative metal-binding motifs may indeed be functionally significant.

In phage T3, substitution of the histidine residues within the putative metal-binding motif (H344-X₂-H347) of the large terminase subunit gp19 (H344D and H347R) resulted in a loss of DNA-packaging activity and/or terminase activity (Morita *et al.*, 1994); substitution at a second histidine pair (H550-X₂-H553) did not have any effect on function. The data from our study are perhaps the most compelling, since a dramatic loss of both the terminase and the DNA-packaging functions was observed by an H \rightarrow R conversion at two histidine residues (H414 of the H411-X₂-H414 pair and the H436 of the H430-X₅-H436 pair) within the histidine-rich region of gp17. One can argue that the substitutions resulted in a major structural or folding perturbation resulting in a non-functional protein. Indeed, such an argument can be made for virtually any amino acid substitution and, clearly, should be a consideration in the functional analysis of any mutant protein. But, the matrix tables developed by Argos and co-workers suggest that these are relatively conservative substitutions from the protein structural standpoint (Bordo & Argos, 1991; Mehta *et al.*, 1995). In addition, from the suppressor data, it is apparent that substitution of the H436 residue with any of the 12 amino acids was not tolerated. These data would argue against a hypothesis that is based purely on protein structural perturbation. Loss of function is most likely due to perturbation of a higher-order structure, such as the metal-binding motif. Also worthy of note is the fact that even the H436C (as in the *E. coli*-Cys suppressor background), which is considered a conservative substitution that is expected to preserve the functional metal-binding motif, was not tolerated. This means that, though cysteine can functionally substitute for histidine, other perturbations arising from the replacement of a bulky imidazole ring with a short aliphatic side-chain can result in a non-functional motif. This would further argue that a precise spatial organization of the metal-terminase interactions may be required for function (Clarke & Yuan, 1995). It should be noted, however, that a catalytic role for the histidine residues, not necessarily through a metal-binding motif, cannot be excluded at this stage.

The proposed metal-binding motif in phage T4, however, needs to be refined. Contrary to the expectation that the H382 residue of the proposed H382-X₂-H385-X₁₆-C402-X₈-H411-X₂-H414 motif would be essential for gp17 function, a H382R mutation had no effect on either the terminase or the DNA-packaging functions. On the other hand, substitutions at the H414 residue within the proposed metal-binding motif, and at the H436 residue that is just downstream to the proposed motif, resulted in a loss of function (H430R resulted in a partial loss of function). These data suggest that the sequence H411-X₂-H414-X₁₅-H430-X₅-H436 rep-

resents a minimal metal-binding motif in gp17. In addition, the upstream C402 may also be a part of this motif. More extensive probing is necessary to precisely define the residues that maintain a functional metal-terminase complex.

With regard to the metal, Guo *et al.* (1987) proposed that Mg^{2+} is the metal ion that interacts with the metal-binding sites in terminases. Their proposal is primarily based on the fact that the $\phi 29$ terminase (and subsequently phage T3 terminase, Morita *et al.*, 1994) can be denatured and renatured in the absence of any externally added metal other than Mg^{2+} (P. Guo, personal communication). But, unless more precise experiments are done to eliminate trace amounts of contaminating metals from the reaction components, this proposal must be viewed with caution (in general, the metal-binding sites are known to be high-affinity binding sites; for example, the dissociation constant for Zn interaction with a tetrahedral metal-binding site is of the order of 10^{-8} M; Regan, 1993). Furthermore, structural studies on a number of ATP-binding proteins show that Mg^{2+} is complexed to protein as an ATP- Mg^{2+} complex *via* interactions with the phosphate groups of bound ATP and the acidic side-chain of the aspartic acid residue(s) (or the carbonyl moiety of the peptide backbone), but not *via* histidine or cysteine (Story & Steitz, 1992; L. Regan, personal communication). On the other hand, it is now well established that histidine and cysteine interact primarily with metals such as Zn, suggesting that the metal-binding site in terminases may indeed be a Zn-binding site. In view of these considerations, a careful analysis of the highly purified terminases by atomic absorption spectroscopy and related techniques will have to be performed in order to identify the metal ion involved in terminase structure and function.

The role of the putative metal-binding motif

Although elegant genetic approaches by Feiss and colleagues (Frackman *et al.*, 1984, 1985; Feiss *et al.*, 1985; Yeo & Feiss, 1995), and more recently by Black (1995) and Morita *et al.* (1995), attributed different functions of terminase to different parts of the polypeptide chain, such a functional dissection may be limited to the subunit interaction sites, e.g. the C terminus of gpNuI interacting with the N terminus of gpA, the C terminus of gpA interacting with the portal vertex protein, etc. But, with regard to catalysis, the data presented here and elsewhere would argue that the functional sites responsible are more complex, and very likely require communication among different sites (Morita *et al.*, 1994; Catalano *et al.*, 1995; Hwang & Feiss, 1996). Two alternative hypotheses, which are not mutually exclusive, are consistent with the functional role of the putative metal-binding motif. First, the proposed metal-binding motif may be required for an interaction that is fundamental to gp17 function; for example, binding to DNA. Perturbation of such an interaction would result in a

loss of both the terminase and the DNA-packaging functions. Alternatively, the metal-binding motif may be required to maintain a precise structural alignment of terminase functional sites for communication and catalysis. This would imply that the functional sites in the phage terminases are not "independent", i.e. they require communication during the DNA-packaging process. One possibility is that the terminase alternates among different conformations, which would dictate which function(s) of the multifunctional complex is expressed (or suppressed). Indeed, there is evidence for ATP-dependent conformational states in phage T3 terminase (Morita *et al.*, 1994). The metal-terminase interactions, by maintaining a precisely aligned protein structure, may allow such dynamic conformational changes. Perturbation of these interactions may result in a structurally unstable and functionally defective terminase. Considering the complex functional interactions required for the DNA-packaging process, this is a strong possibility. Further probing of gp17 structure and function is underway to understand the molecular mechanism of action of this very interesting phage protein.

Materials and Methods

Bacteria, phage, and plasmids

E. coli B40 (*su*⁺) was used as the amber suppressor for preparation of phage stocks. *E. coli* P301 (*sup*⁻) was used as the non-suppressor strain for preparation of packaging extracts. *E. coli* VR34 (NS3529 λ imm λ LP1 λ imm434-P1:Cre⁺) was used for plating packaging reaction mixtures (Rao *et al.*, 1992).

E. coli strains BL21, BL21 (DE3), BL21 (DE3) pLys-S and BL21 (DE3) pLys-E (Novagen) were used as the host strains for pET plasmids carrying g17 constructs. Construction of these strains, and conditions for growth and IPTG induction, were as described (Studier *et al.*, 1990). *E. coli* BL21 lacks the T7 RNA polymerase gene and is therefore a non-expression strain. It was used for long-term stable maintenance of all the recombinant constructs. It was also the strain used for the first transformation of any T7 recombinant construct. After screening and ascertaining the recombinant construct, miniprep DNA was prepared and the DNA was then transformed into an expression strain, either the BL21 (DE3) or the BL21 (DE3) pLys-S.

The *E. coli* suppressor strains (Kleina *et al.*, 1990) carrying the tRNA suppressor for each of the amino acids Ala, Arg, Cys, Gln, Glu, Gly, His, Leu, Lys, Phe, Pro, Ser and Tyr were kindly provided by Dr Jeffrey Miller, University of California, Los Angeles. The *E. coli*-His and *E. coli*-Lys suppressor strains were used for rescuing the T4.H436am and the T4.K166am phage, respectively, and for preparation of the mutant phage stocks. Lysogenization of the suppressor strains to (DE3) pLys-S phenotype were done using the Novagen lysogenization kit (Studier *et al.*, 1990).

Wild-type T4, 17amNG178, 17amA465.del.rIIA phage stocks were prepared in this laboratory.

The phage T7 vector pET 9D (*kan*^r) (Novagen Inc.), which has an *Nco*I site and a *Bam*HI site downstream from the T7 promoter was used for construction of g17

recombinants (Studier *et al.*, 1990). pR 17-8 was constructed by inserting the complete g17 coding sequence with engineered *NcoI* ends into the *NcoI* site of pET 9D (Bhattacharyya & Rao, 1993). pR 17-8 was used as the basic plasmid for construction of all the mutant plasmids.

Primers

PCR primers

(I) 5'-TGGACTGCTGCTGTCGAAG-3' (nucleotides 892 to 910); (II) 5'-CGCGGATCCGTTATACCATIGA-CATACCATGAG-3' (1833 to 1811; the italicized nucleotides represent the tag sequence added to the 5'-end of the oligonucleotide for efficient cutting at the adjacent *Bam*HI sequence shown in bold). These primers were used in all the amplification experiments in which the 3' g17 fragment (942 bp) was amplified. The amplified DNA was then digested with *Pst*I and *Bam*HI, and the 770 bp *Pst*I-*Bam*HI fragment was ligated with the *Pst*I-*Bam*HI linearized and dephosphorylated pR 17-8 large fragment (see below). (III) 5'-CATGCCATGGAAGGCTTGATATAAACAAC-3'; (g16, 1 to 25); (IV) 5'-CATGCCATGGTATTTATACCATGACATAC-CATGAGA-3' (1836 to 1810). These primers were used in some experiments to amplify the entire DNA fragment spanning both the genes 16 and 17. As with the primer II shown above, these primers also have a 5'-tag sequence (italicized nucleotides) for efficient cutting of the adjacent *NcoI* restriction site (shown in bold).

Mutagenesis primers

H436am mutant primer, 5'-GTTTTGCATTCAAA-CACG(t)ATA(t)TCIT(c)AG(t)TTAATTCTACCTGAC-3' (1282 to 1323; creates an *EcoRV* site); E536V mutant primer, 5'-AGGCGTGTCTTGGGCC(g)GCTGT(a)AG AAGGTTAT-3' (1587 to 1617; creates a *Hae*III site); H382R mutant primer, 5'-CCAGATGATCG(a)TGGTTTT-CACCAA-3' (1135 to 1158; removes a *Bcl*I site); H414R mutant primer, TACCACGCTTTA(g)CG(a)TATTATT-GATGTTAC-3' (1228 to 1256; creates a *Sna*BI site); H430R mutant primer, CAGGTTGGTGTGTTTGC-G(a)A(t)TCG(a)AACACTATTCTCATTTA-3' (1273 to 1311; creates a *Pvu*I site); K166am mutant primer, TCGCGCCAGCTCGGTT(a)AG(a)ACCACCGTAGTAG-CT (481 to 513). For creating each mutation by SOE, two complementary mutant primers are needed (see below); the above sequences represent only one of these primer sets (the coding strand primers); the nucleotides in bold represent the mutations introduced at that position, with the wild-type sequences shown in parentheses. Note that some of the mutations represent silent mutations, which were introduced either to create or to abolish an adjacent restriction site as indicated in parentheses.

Sequencing primers

The following primers were used for sequencing the g17 mutants: (I) 5'-CGTACTATCAGCGTGATA-3' (nucleotides 418 to 436); (II) 5'-GGCTCAATGTCTGCG-GAAG (577 to 595); (III) 5'-TGGACTGCTGCTGTC-GAAG-3' (892 to 910); (IV) 5'-GCAGCGTTTGAA-GGGACTT-3' (1060 to 1078); (V) 5'-GATTACCACG-CTTTGCATA-3' (1225 to 1243); (VI) 5'-TACTGGTGTGT-CAGTTGCA-3' (1383 to 1411); (VII) 5'-CATCACC-GAGCGACTATTC-3' (1543 to 1561); (VIII) 5'-GATGA-CATGCGATTAGCATC-3' (1696 to 1715).

Note that the nucleotide numbers in parentheses correspond to the coding sequence of g17 unless otherwise noted.

Standard techniques

DNA mini-preps were prepared either by the procedure of Birnboim & Doly (1979), or by using the RPMI miniprep kit (Bio101). Large-scale plasmid DNA preparation and equilibrium *CsCl* density-gradient purification of plasmid DNA were performed according to procedures described by Maniatis *et al.* (1982). Transformation-competent cells were prepared either by the *CaCl*₂ method (Maniatis *et al.*, 1982), or by the SEM protocol (Inoue *et al.*, 1990). In some experiments, transformation was performed by the electroporation procedure described by Dower *et al.* (1988). The target g17 DNA fragments were amplified either from single plaques, or from purified phage T4 DNA by PCR according to the conditions described by Yap & Rao (1996). The amplified DNA was first purified by agarose gel electrophoresis followed by extraction from sliced agarose using the Qia-gen gel extraction kit. The gel-purified DNA was then used for a variety of manipulations including ligation, site-directed mutagenesis and DNA sequencing. DNA sequencing was performed by cycle sequencing according to the procedure described by Rao (1994). Either the Cyclist™ *Pfu* (exo⁻) sequencing system (Stratagene) or the Fentamole™ DNA sequencing System (Promega) was used to sequence the DNA.

Random mutagenesis of gene 17

The pR17-8 plasmid DNA (20 µg) was added to the mutagenesis reaction mixture containing 250 mM sodium acetate (pH 7.0) and 1 M sodium nitrate and incubated for 40 minutes at room temperature. The mixture was then diluted with 480 µl of TE buffer, 480 µl of 2.5 M sodium acetate (pH 7.0), and 24 µg of yeast tRNA. The mutagenized DNA was then precipitated by adding 2.4 ml of 95% (v/v) ethanol and incubating it at -70°C for one hour. The mixture was centrifuged at 12,000 rpm for 15 minutes, and the pellets were washed twice with 75% ethanol. The mutated DNA was then resuspended in distilled water. The 942 bp 3'-end of g17 was amplified using the PCR primers I and II. The amplified mutant DNA was purified by agarose gel electrophoresis followed by extraction from the sliced agarose using the Qia-gen gel extraction kit.

Site-directed mutagenesis of gene 17

Site-directed mutagenesis was performed using a modified Splicing by Overlap Extension (SOE) strategy described by Chen & Przybyla (1994). The mutagenesis was accomplished in two stages using four primers (Higuchi *et al.*, 1988; Horton *et al.*, 1989).

In the first stage, the two halves of the 942 bp fragment of g17 were amplified. The first half extended from nucleotide 892 to about 20 nucleotides following the mutant site, and the second half extended from about 20 nucleotides preceding the mutant site to the 3'-end of g17 (nucleotide 1833). The first half was amplified using PCR primer I and the non-coding strand mutagenesis primer that corresponds to the mutant sequence and the flanking sequences. The second half is amplified using the coding strand mutagenesis primer and PCR primer II. PCRs were performed using *Pfu* polymerase (Stratagene)

for 25 to 30 cycles in an Ericomp thermal cycler. It was important to use *Pfu* polymerase for mutagenesis because: (i) the *Pfu* enzyme, unlike the *Taq* polymerase, generates fully blunt-ended amplified DNA, thus minimizing errors at the mutagenesis site, and (ii) since the *Pfu* enzyme has a proof-reading 3' → 5' exonuclease activity, the frequency of error incorporation is 15 to 30-fold less than that for the *Taq* polymerase (Lundberg *et al.*, 1991). Each PCR cycle consisted of: (i) denaturation of DNA at 95°C for 30 seconds, (ii) annealing of the primers at 45°C for 30 seconds and (iii) extension at 72°C for 45 seconds. The PCR products were purified by agarose gel electrophoresis followed by extraction of DNA from sliced agarose using the Qiagen gel extraction kit.

In the second stage, the two halves of g17 are "stitched" together to reconstitute the full-length g17 sequence. Due to the use of complementary mutant primers in the first stage, the products will have a stretch of identical sequence (30 to 40 nucleotides) flanking the mutation site. To stitch the two halves, both the half-length products were mixed and PCRs were performed in the absence of any primer. The template strands are then forced to anneal through the overlapping ends to prime DNA synthesis in both the directions and generate the stitched product. PCR primers I and II were then added to the reaction mix to amplify the full-length product. The PCR product was electrophoresed on an 0.8% agarose gel followed by extraction of DNA from sliced agarose using the Qiagen gel extraction kit.

Cloning the mutant fragments

The amplified 942 bp fragment either after random mutagenesis or site-directed mutagenesis was double-digested with *Bam*HI and *Pst*I. The DNA was electrophoresed on an 0.8% agarose gel, and the large 770 bp fragment was extracted from the gel slice. To replace the wild-type g17 with the mutated fragment, pR17-8 was double-digested with *Bam*HI and *Pst*I. The DNA was dephosphorylated with alkaline phosphatase (Promega), and the large fragment was gel-purified. The mutant PCR-fragment was ligated with the dephosphorylated pR17-8 fragment using T4 DNA ligase (Promega), and the ligated product was first transformed into *E. coli* BL21. Individual transformants were analyzed for the presence of appropriate insert. Mini-prep plasmid DNA was prepared from the desired transformant(s) and was re-transformed into the expression strain BL21 (DE3) or BL21 (DE3) pLys-S.

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